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Methane Prediction by Nutrient Profiles in Ruminal Continuous Cultures Fed an All Forage Diet of Bermudagrass or Annual Ryegrass

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ABSTRACT

Extensive research has been done on the effect of diet on rumen methane (CH_4) production, and on developing equations to accurately predict CH_4 in cattle. However, the majority of this research has been gathered from feedlot cattle or cattle fed a total mixed ration (TMR). To date, no studies have examined nutrient correlations with CH_4 when feeding an all pasture diet of warm season or cool season grasses. This study included two in vitro experiments, one with a warm season forage and one with a cool season forage to see which nutrient characteristics of each forage best correlated with CH_4 production. Rumen microorganisms from a lactating Holstein cow were incubated in dual-flow ruminal continuous cultures for 7 days and thirty g of either Tifton 85 bermudagrass in experiment 1 or Marshall Annual Ryegrass in experiment 2 at 5 different days regrowth (14 d, 21 d, 28 d, 35 d, and 42 d) were fed twice daily in equal amounts. Methane concentrations were measured hourly to determine differences in CH_4 production with time, forage species and regrowth. In experiment 1, feeding bermudagrass at 28 d regrowth resulted in CH_4 production (32.13 mmol/d) which was higher than all others except for 35 d. The three nutrients included in the forward regression, were starch, sugar, and acid detergent lignin (ADL). In experiment 2, feeding annual ryegrass at 21 d regrowth produced the highest amount of CH_4 (17.21 mmol/d) compared to all other days regrowth. The three nutrients included in the forward regression

were starch, ADL, and hemicellulose (HC). For both experiments, measured values were lower than predicted ones from equations. These experiments conclude that starch is the strongest predictor of CH_4 in grazed forages but other predictors may vary based on grass type.

DEDICATION

I would like to dedicate this work to my parents Janet and Greg Young. Without their unconditional love and support I wouldn't have become the person I am today. Also to all my other family and friends who have stayed by my side through thick and thin, and helped me to stay focused and achieve my goals.

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TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

REVIEW OF THE LITERATURE

GRAZING INTENSIVE DAIRY SYSTEMS

Pasture grazing plays a role in most dairy cattle operations. Feeding systems that rely on grazing for the majority of dairy cattle diets are called grazing intensive dairies (GiDs). Although there is much variation between grazing systems on different farms, the intensive grazing technique typically involves intensive grazing of cattle for a given period of time on all of the pasture or on a portion of the pasture. In some grazing systems, cattle can be continuously grazed on pasture without rotation. During rotational grazing, cattle are grazed in divided paddocks, typically only on a portion of the pasture at any given time. Cattle are then moved to a different paddock to allow uniform growth and recovery of the grass (Hanson et al., 1998). The amount of time that cattle spend grazing in a given paddock depends on the amount of paddocks in use, the stocking density of the cattle, and the forage yield and quality. Grazing time typically ranges from several hours in an intense time-controlled rotational grazing system (Hart el al., 1993) to seven days in a regular rotational grazing system. There have been mixed thoughts about the benefits of continuous vs. rotational grazing.

Effects of continuous vs. rotational grazing on cattle production have been mixed. Some studies have shown that there is no difference in milk production in continuous vs. rotational dairy grazing systems (Davis and Pratt, 1956) while some beef

cattle studies claim that there is a slight increase in average weight gain with continuous grazing (Rogler, 1951; McIlvain and Savage, 1951). However, there is agreement that rotational grazing is beneficial to density and vigor of vegetation. Grazing systems are more favorable than the traditional total confinement systems in the southeast due to the longer grazing season. The most interest in grazing systems has been with dairy operations having fewer than 100 cattle allowing more pasture area per cow (Parker et al., 1992). These smaller farms are also subjected to more financial stress, so the net profit per cow is more important (Parker et al., 1992). Some commonly used forage in GiDs include tall fescue, bermudagrass, ryegrass, switch grass, brome grass, alfalfa, and clover. Typically, a warm-season grass such as bermudagrass is over seeded with a cool-season grass such as ryegrass so that cattle can be grazed on the pasture year round.

Recent Trends in Cattle Feeding Systems

In 2011, the Southeast region of the United States had the lowest amount of milk marketed by producers compared to most other regions in the US (USDA, 2012). The Southeastern US climate is characterized by a longer hot season, thus making it difficult for dairy cattle to lower body temperatures. High ambient temperatures combined with high levels of metabolic heat produced by the cow cause a decrease in intake and therefore milk production (West, 2003). Therefore, dairies in this region have suffered losses due to heat stress, thus decreasing competitiveness and production of

southeastern dairies (West, 2003). This results in high transportation costs for the dairy products and thus high dairy prices for consumers.

Total Confinement Dairies (TCDs), are typically more popular nationwide, especially in the north and midwest regions due to a longer cold season in those areas. In these systems, cattle are kept in a confined area and fed a total mixed ration (TMR) of silage, grain and hay. Grain and silage are not favorable to grow in the southeast due to less yield, lower soil quality and mycotoxins (Gerrish, 2004). Corn silage contains nearly twice the dry matter (DM) yield compared to an intensive grazed pasture so feeding a TMR optimizes nutrition and production per cow better than grazing can. As a result, overall milk production is typically higher per cow in cattle raised in TCDs. However the cheaper production cost advantage of GiDs compensates for the production advantage of TCDs (Hanson et al., 1998). Interest in GiDs has increased in the southeast over the last decade. Grazing intensive dairy system are more favorable in the southeast due to the warmer weather, and therefore longer grazing season. Although cattle in GiDs produce less milk than TCDs, GiDs are desirable due to reduced operation and machinery costs.

Benefits Associated with GiDs

Factors such as reduced production costs, environmental friendliness and improved quality of life have all previously been identified as benefits to grazing operations (Gerrish, 2004). A study done by Parker et al. (1992) on a Pennsylvania dairy

farm compared management and economic implications of intensive grazing vs. the traditional total confinement system by using spreadsheet models and a 80-ha case farm with 53 cattle, and 43 replacements and a herd average of 6800 kg per year per cow. They found that intensive grazing dairies resulted in reduced costs for total operating expenses. This is mostly due to harvesting and labor costs that are typically associated with TCDs. Total confinement systems tend to have more costs associated with crop and forage production due to machinery and manual labor costs. It was estimated that profitability was approximately $121 more per cow when using the grazing system (Parker et al., 1992). Hanson et al. (1998) conducted a study which compared profitability of "moderate" grazing systems to that of extensive grazing systems and traditional confinement systems. A moderate grazing system was defined as grazing cattle kept on a pasture for 7 days or less before rotating them to a new pasture, relying on grazing for 50% or more of forage needs, and having more than 4 paddocks in use. Moderate grazing was found to be more profitable as compared to extensive grazing systems and conventional systems.

However, a concern is variability in forage growth between seasons and years, especially during drought. Rations that include intensive grazing must be carefully monitored in order to make sure that nutrients are balanced. Otherwise reproductive health and milk quality can decrease (Hanson et al., 1998). Rotational grazing as well as availability of alternative feeding sources such as silage and grain supplementation may help to alleviate these problems (Parker et al., 1992). Other advantages to intensive

grazing systems include environmental sustainability through reduced use of fertilizers and chemicals, and reduced lameness and hoof damage caused from concrete floors in TCDs (Hanson et al., 1998). Since cattle spend more time on pasture, more of their manure is deposited in the grazing paddock. This results in more organic material being deposited in the soil, and reduces the need for fertilizers. Data from a liquid manure hauling truck company in Alberta reported that 30 ton trucks charge approximately $80 per hour for transport while operation of a manure pipeline cost approximately $50 per hour (Ghafoori et al., 2007).

Furthermore, milk from cattle raised on GiDs has been associated with health benefits because studies have shown that pasture grazing leads to increased levels of omega-3 fatty acids and conjugated-linoleic acids (CLA) in milk when compared to a TMR diet (Kelly et al., 1998; Dhiman et al., 1999; Kraft et al., 2003). Conjugated linoleic acids are derivatives of linoleic acid that come from the incomplete biohydrogenation of polyunsaturated fatty acids in the rumen, or from mammary gland derivatives of biohydrogenation (Kelly et al., 1998). This process involves isomerization and then successive reductions to form stearic acid (Grinardi et al., 2000) Ideal levels of CLA require optimum substrate availability and fermentation (Kraft et al., 2003). There are 28 possible isomers of CLA which differ in the position of the double bond and the configuration around the double bond (*cis* or *trans*). The most common type found in dairy products is 18:2 *cis*9*trans*11, more commonly known as rumenic acid which is known to be an anti-carcinogen (Tvrzicka et al., 2011). Another type is 18:2

*trans*10*cis*12, which has anti-obesity effects. Conjugated-linoleic acids have been found to have antioxidant properties as well (Tvrzicka et al., 2011). In studying mice, CLA has been shown to increase bone mass and protect the body against bone loss (Park and Pariza, 2008; Rahmen et al., 2007).

GiDs Effects on the Environment

Grazing intensive dairies can affect the environment in terms of soil composition, runoff and gas emissions. Nutrients are directly deposited back into the soil in the form of manure and urine, and some of these nutrients are taken up by plants once again (White et al., 2001). White et al. (2001) measured distribution of urine and feces from dairy cattle in a rotational grazing system. Data showed that urine and fecal deposition corresponded strongly to the amount of time that cattle spent in a given area. For example, urine and fecal deposition was more concentrated around the water tank than anywhere else in the pasture. Handling cattle quietly and efficiently can minimize fecal and urine deposition in the facilities, which in turn maximizes deposition in the paddock. Grazing cattle play a vital role in nutrient cycling in pastures. Cattle consume grasses to produce meat and milk, and redeposit organic material in the soil via manure. In a GiD, cattle spend more than 90% of their time on pasture and deposit more than 90% of their manure on the pasture. In contrast, cattle in TCDs deposit most of their manure on the floor of covered facilities, which is then transported to only a portion of the land as fertilizer (White et al., 2001).

Increased manure deposition from grazing leads to increased carbon sequestration in the soil, or the capture and long-term storage of atmospheric carbon dioxide. Carbon soil stocks decrease as soil depth increases. Farms that have practiced intensive grazing for longer periods of time have a dark upper soil layer. This is likely due to increased manure deposition on the pasture over time. Unpublished research done at the University of Georgia showed a GiD chronosequence from 0-3 years after conversion from row crop to GiD. A soil chronosequence is a sequence of soils that changes gradually with time. In this case, the chronosequence were farms of similar climate and soil type but had been practicing intensive grazing for different periods of time. Data from a preliminary study in Wrens, GA experienced an increase in soil organic matter of 0.44 $\pm$0.08% between 2007 and 2010 (Frazluebbers et al., 2000, 2001). Application of animal manure to fields contributes to better soil quality, and provides an alternative to application of traditional fertilizer and chemicals (De Freitas et al., 2003). Soils treated with cattle manure have been reported to have higher levels of microbial activity. Cattle manure is higher in organic carbon and lower in nitrogen, which may influence the biomass and result in higher microbial activity (De Freitas et al., 2003).

Grazing cattle depositing manure on the ground has the potential to not only increase beneficial soil organic matter but also reduce runoff pollution. Goetz (1999) found that runoff pollution was greater in conventional dairy systems than in grazing systems. However, overgrazing could result in potential groundwater contamination due

to exposed ground cover, high stocking rates, and excessive fertilization (Owens et al., 1982).

Finally, GiDs have been thought to increase the amounts of greenhouse gases in the atmosphere, especially methane (CH_4) but also nitrous oxide. The CH_4 production in cattle is closely correlated with the diet fed, and the digestibility of the feed. So it is predicted that grazing cattle on forages, particularly early maturity forages causes an increase the amount of CH_4 produced by the cow, which increases the amount of CH_4 in the environment. This is due to their high digestibility which increases the amount of substrates for methanogenesis. In order to better understand GiDs' contribution to CH_4 in the environment, it is necessary to understand digestion and CH_4 production in the cow.

RUMINANT DIGESTION

Dairy cattle are part of a group of animals called ruminants, which are characterized as having four compartments to their stomach: the reticulum, the rumen, the omasum and the abomasum. Following fermentation and digestion, feed passes through the small intestine which is composed of the duodenum, jejunum and ileum (Van Soest, 1994). The largest of the four stomach compartments is the rumen, which serves as a large fermentation vat for feed. Cattle consume feed which is broken down into smaller fragments by mastication followed by swallowing and regurgitation of the feed called rumination. This process incorporates saliva to maintain an adequate pH between 6.0 and 7.0 in the rumen (Van Soest, 1994). The rumen is inhabited by a community of microbes including bacteria, fungi, and protozoa. These microbes live in a symbiotic relationship with the cow, and ferment feed to produce volatile fatty acids (VFA) (Van Soest, 1994). These VFA are an important source of energy for the cow and can serve as precursors for milk production. The cow's digestive system provides the warm anaerobic environment and constant food supply that these microorganisms need to survive. These microorganisms have the unique ability to break down the beta 1-4 linkages in cellulose that are indigestible to non-ruminant creatures, except through hind-gut fermentation. This allows the cow to make use of feedstuffs that would otherwise be unusable, and is the reason why producers are able to feed high levels of forages to ruminants (Van Soest, 1994).

Feeding Forages to Ruminants

Adequate forage amount in the diet is necessary in order to maintain proper rumen function in the dairy cow. Forages generally make up anywhere from 50% to 100% of the dairy cow's diet depending on the feeding system. Starches and sugars are more rapidly and completely degraded in the rumen than cellulose due to the alpha linkage between the glucose monomers instead of the beta linkage (Van Soest, 1994). Less forage and smaller forage particle size in the diet means that the cow spends less time ruminating and incorporating saliva in with the feed. This results in quick passage, and a low pH in the rumen and therefore decreased activity of microbes. Feeding too much grain can result in an acidotic state, which lowers ruminal pH and limits growth of cellulolytics. Feeding forages encourages mastication and incorporation of saliva, thus regulating ruminal pH.

A higher concentration of forages in the diet results in more acetate produced during fermentation. A higher concentration of grains in the diet results in more propionate (Bauman et al., 1971). This shift in VFA proportions is directly related to the diet's effects on the rumen microbes. Grains have a higher digestible energy, which explains why diets with a higher grain concentration are associated with higher milk production. However, since feeding forages increases acetate production which is a precursor for milk fat, feeding forages could result in increased milk fat percentage.

Farmers are paid more based on milk fat percent, so this could potentially make up for some of the lost profits due to lower milk production.

Influence of Forage Quality

Another forage characteristic that influences consumption and digestion by the cow is the quality of the forage fed. Forage quality can be defined as "the physical and chemical characteristics of a forage that make it valuable to animals as a source of nutrients and well-being"(Balasko and Nelson, 2003). Forage intake depends heavily on forage quality. Palatability is one factor that affects intake. Animals tend to prefer forages with softer leaves and stems such as ryegrass (Balasko and Nelson, 2003) because they are easier to chew. The stage of maturity is found to influence palatability, digestibility and crude protein levels (Ball et al., 2002). Mature forages have a "woody" texture, are harder to chew and are more slowly digestible. These forages typically have a slower passage rate through the rumen causing a decrease in feed intake and production.

Lignin has a negative effect on forage quality since it becomes cross-linked with the cellulose and hemicellulose in the cell wall thus giving it the "woody" texture. This helps to protect the plant against physical damage and disease but makes the cell wall less digestible (Balasko and Nelson, 2003). Lignin is, for the most part, difficult for the rumen microbes to break down, thus decreasing the digestibility of the plant. Lignin content increases with plant maturity so waiting too long to graze animals on a pasture

can decrease animal performance through lack of digestibility. On the other hand, protein levels are thought to be inversely proportional to the maturity of the forage (Hill et al., 1995).

Influence of Forage Type

In addition to forage maturity, different types of forages also influence cattle performance. Forage type influences the nutrient profile, which can affect the digestibility, intake and weight gain. For example, Beever et al. (1986) found that feeding ryegrass increased both organic matter intake and propionate levels compared to feeding white clover. In a study comparing performance of beef cows consuming bermudagrass over seeded with ryegrass, arrowleaf clover, and crimson clover, weight gain was found to increase when over seeding with clover and a ryegrass and clover mixture (Hoveland et al., 1978). Galloway et al. (1993) also found that orchardgrass has a higher NDF and total tract digestibility than bermudagrass. In comparing ryegrass vs. bermudagrass, ryegrass tends to have higher sugar content and lignifies more slowly than bermudagrass. Warm season grasses like bermudagrass tend to have higher fiber and lignin and lower protein than cool-season forages like annual ryegrass (Ball et al., 2003).

Forage type can also have effects on milk in dairy cattle. Limited information is available about the effects of forage type on milk composition but there have been some studies correlating milk fatty acid content with the fatty acid composition of the

plant consumed. In temperate countries, fresh grass contains about 1-3% fatty acid with the highest fatty acid concentration being observed in the fall and spring. Cattle grazed on pasture have also been found to have higher levels of linolenic acid in their milk fat compared with cows fed silages or corn (Chilliard et al.,2001). When cattle are fed a diet rich in alpha linoleic acid, there is a higher concentration of CLA in the milk. Kraft et al. (2003) compared differences in CLA isomer distribution between cattle grazed in the Alps verses those grazed in indoor feeding systems, and found that cattle grazed in the Alps had a higher concentration of eicosapentaenoic acid (EPA) and a lower concentration of arachadonic acid in milk but had overall higher concentrations of polyunsaturated fatty acids due to the higher concentration of alpha-linolenic acid found in mountain pasture. Although Decaen et al. (1970) found no differences in linoleic and linolenic concentrations in milk between different forage types (ryegrass, alfalfa, or orchardgrass), there have been differences observed fatty acid concentrations in milk between grazed dairy cattle and those in a traditional confinement system. Dhiman et al. (1999) found that cattle that had one-third or two-thirds of their diet supplemented with pasture had increased amount of CLA with the amount of forage fed. Cows grazed only on pasture had 500% more linoleic acid in milk than cows fed a corn silage based diet supplemented with corn oil. Kelly et al. (1998) found that conjugated linoleic acid concentrations from cattle consuming forages from a GiD were nearly double of those consuming a traditional TMR. Similarly, a study by Vanhatalo et al. (2007) tested the effects of feeding a timothy meadow fescue silage based diet or red

clover silage based diet of early or late maturity on fatty acid composition in milk. Feeding red clover increased levels of monounsaturated, and polyunsaturated fatty acids; in particular linolenic acid content in milk. Greater increases in polyunsaturated fatty acids were seen when cattle consumed red clover swards of earlier maturity.

Rumen Continuous Cultures

Although feeding forages directly to ruminants is the most accurate way to measure digestion and other effects on cattle and their surrounding environment, trials such as these can be time consuming and costly. Artificial rumens, or rumen continuous cultures offer a less expensive alternative since less feed and labor is involved than in a live animal study. In an artificial rumen, rumen fluid is typically collected from a fistulated cow, strained through cheesecloth, and combined with buffer prior to adding it to the continuous culture. Feed can then be added daily to be digested by ruminal bacteria.

The design of some continuous cultures allows for natural stratification of feed particles as seen in an actual rumen having a “mat” maintained on the top, liquid layer in the middle and another particle layer on the bottom. This occurs through selection of a suitable stirring speed. Buffer is continuously infused into the rumen fluid in order to simulate saliva, which maintains an adequate ruminal pH of 6.0-6.5. The normal temperature in the rumen is approximately 39° C and so the cultures are kept heated at 39° C to maximize fermentation. Culture contents are also kept anaerobic through

continuous flow of CO_2 or nitrogen (N) in the culture to displace any oxygen (O_2) that may enter. There is also an overflow port and collection flask that allows the mixture of feed, buffer and rumen fluid to be pushed out and collected as buffer is pumped in. Overflow samples represent completed fermentation in the rumen, and can later be used for analyses.

Ruminal continuous cultures were developed and used in the late 1950's and early 1960's, and there have been several different devices used for this purpose (Slyter et al., 1964; Eun et al., 2004; Teather and Sauer, 1988). Unlike in batch cultures, continuous cultures allows for more sampling of ruminal fermentation and turnover as well as addition of new feed every day. Ruminal continuous cultures can measure digestibility of feed from the difference between DM input and DM output. In addition, CH_4 gas concentration has also been measured in these cultures from headspace sample (Eun et al., 2004). This way it is possible to see changes in CH_4 production with feed type and maturity. While this method is accurate, it is time-consuming and only gives spot samples instead of continuous changes in CH_4 production over time.

METHANE PRODUCTION

On average, CH_4 makes up the second-largest percentage of gas produced in the rumen (24-27%). Cattle can produce up to 44 kg of CH_4 annually per cow (McAllister et al., 1996). Fermentative bacteria and protozoa in the rumen break down simple sugars to CO_2, acetate and H_2 in addition to producing some propionate, butyrate, ethanol and lactate. Finally, the methanogenic bacteria take the CO_2, acetate and H_2 and catabolize them to make CO_2 and CH_4 (Bryant, 1979). The most common pathway of methanogenesis is one in which CO_2 is reduced to CH_4 in the presence of hydrogen ($CO_2 + 4H_2 = CH_4 + 2H_2O$). Carbon dioxide is converted to CH_4 through four reductive intermediates and six coenzymes. Carbon dioxide is fixed with methanofuran (MFR) to produce the intermediate formyl-MFR. The formyl group is transferred to tetrahydromethanopterin (H_4MPT), which is a carrier for the intermediates methenyl, methylenyl and methyl. Methenyl-H_4MPT is reduced to methylenyl-H_4MPT and methylenyl-H_4MPT is reduced to methyl-H_4MPT, and all of these reactions are carried out by coenzyme F_{420} (Ferry, 1992). Then, the methyl group is transferred to coemzyme M (HS-CoM). This is then reduced to CH_4 by methyl coenzyme reductase complex composed of $F_{430,}$ ATP, 7-mercaptoheptonoyltheorine (HS-HTP), and FAD (McAllister et al., 1996). The microbial species that produce CH_4 are called methanogens, in particular, *Methanobrevibacter ruminantium* produces most of the CH_4 in the rumen. Other common methanogenic species include *Methanobrevibacter thaueri, Methanobrevibacter millerae, Methanobrevibacter smithii,* and *Methanobrevibacter*

olleyae (Danielsson et al., 2012). Methane production acts as a hydrogen sink since its' production uses H_2. Propionate production also acts as a hydrogen sink, which is why an increased proportion of propionate being produced results in decreased CH_4 production.

Importance of CH_4 Production in Rumen Fermentation

Although CH_4 has been a concern as a greenhouse gas, its production is necessary to rumen fermentation. During microbial glycolysis, carbohydrates are broken down to simple sugars. These simple sugars are then turned to pyruvate and then to acetate, propionate or butyrate. Taking sugars to pyruvate requires NAD+ as a cofactor, where it is taken to NADH. Since NAD+ is needed as a cofactor for microbial glycolysis, increased levels keep glycolysis and therefore fermentation moving forward. There are high levels of H_2 in the rumen keeping it a highly reduced environment. Both propionate production and CH_4 production act as hydrogen sinks to decrease the levels of H_2 to regenerate NAD+. Another way that cattle get rid of CH_4 from the body is via belching. Belching is necessary, and failure to do so may result in bloating in the cow. However, the main method adapted by cattle is that eructation diverts methane to the lungs so that it can then be exhaled (Van Soest, 1994). This release of gas causes an increase in CH_4 in the atmosphere.

CH_4 in the Environment

Methane is one of the most abundant gases in the atmosphere, and it can come from either biogenic living sources or from abiogenic nonliving sources. Methane is 21 times more potent than CO_2 in its ability to trap heat in the atmosphere (Kebreab et al., 2008). Methane concentration has been increasing by about 1% per year over the last couple of centuries (Cicerone and Oremland, 1988). Furthermore, livestock account for 35-40% of the global anthropogenic emissions of CH_4 via enteric fermentation and manure (Steinfeld et al., 2006). Methane is the dominant gas produced by anaerobic degradation of organic material. This is done primarily by bacteria; methanogens in particular (Conrad, 1996). In some ecosystems protozoa may contribute as well (Bryant, 1979). Methane can also be consumed by bacteria living naturally in the environment called methanotrophs. These bacteria are widely abundant, and are present in both soil and water environment where there is CH_4 and O_2. When O_2 is present, methanotrophs combine O_2 and CH_4 to form formaldehyde which is incorporated into organic compounds.

Methane production has become a topic of interest in global warming in recent years. Dairy cattle produce approximately 120 L/d CH_4 while beef cattle produce approximately 80 L/d CH_4 due to higher grain-based diets (Phillips, 2010). Agriculture in the United States is thought to contribute 8% of the total greenhouse gas emissions in

the US and is the second largest CH_4 source in the United States (US EPA, 2007) with landfills being the largest.

Methane emissions can come from direct eructation as well as from field deposited manure and lagoons. Increasing time on pasture results in more manure deposited in the field. However, the CH_4 production from deposited manure is thought to be significantly lower than eructed CH_4, and CH_4 production from lagoons is thought to be higher than field-deposited manure. Lagoons are man-made basins filled with cattle waste that undergo anaerobic respiration. Amon et al. (2006) found that CH_4 emission from deposited manure is three times lower than emissions from lagoons when measured on a surface area basis.

Reducing CH_4 Emissions

Due to the rising concern of CH_4 in the environment and since it represents energy loss in the rumen, research has been done on ways to reduce CH_4 emissions. Cattle lose around 6% of their digested energy as eructed CH_4. Researchers have looked at ways at reducing CH_4 emissions by altering feeding patterns. Some causes of variation in CH_4 emission include level of intake, supplementation of ionophores, type of carbohydrate, forage maturity, lipid supplementation, grain to forage ratio and feed processing (Johnson and Johnson, 1995). Less intake results in less substrates for fermentation and therefore CH_4 production. Research in reduction of CH_4 has been done with the use of compounds that are toxic to methanogens such as chlorinated CH_4

analogues or ionophores such as monensin that inhibit CH_4 production from methanogens. McGinn et al. (2004) found that monensin supplementation decreased CH_4 emissions when included in the diet. However, Waghom et al. (2008) found that using monensin-controlled-release capsules did not decrease CH_4 emissions due to inadequate performance of the delivery device. Ionophores inhibit CH_4 production by causing a shift to more propionate production and less formate and acetate production. Since propionate production uses H_2 while acetate production creates it, there is less free H_2 available. The decrease in the amount of substrates for the methanogens results in decreased CH_4 production (McAllister et al., 1996).

In addition, CH_4 production from cattle is also influenced by the quality and type of the feed components; in particular highly soluble components such as sugars and carbohydrates are quickly fermented to CO_2 and H_2 driving methanogenesis forward (McAllister et al., 1996). However, highly soluble carbohydrates are also believed to promote production of propionate, which is correlated with lower CH_4 production (Van Kessel and Russell, 1996). Forages are degraded more slowly in the rumen than concentrates thus reducing the amounts of available CO_2 and H_2 from feeds as CH_4 substrates. However, forages also produce higher levels of acetate which is also a substrate for CH_4 production so this may help to drive methanogenesis forward as well (Kebreab et al., 2008). Differences in CH_4 emissions can also be seen between different species of forages. Bash et al. (2012) found differences in CH_4 production when

incubating various leguminous and non-leguminous shrubs native to Australia and Ghana *in vitro.*

Staerfl et al. (2012) fed high water soluble carbohydrate ryegrasses (WSC) and low (WSC) ryegrasses to look at effects on CH_4 production. Methane production was similar when both types of ryegrasses were fed, but the high WSC ryegrass was found to have lower crude protein content. Methane production has been found to be directly correlated with protein content in the plant and inversely correlated with lignin content in the plant. At earlier maturity, forages are more digestible, and so more H_2 and CO_2 is produced than when cattle consume later maturity forages. As forages mature, percent lignin increases, thus decreasing digestibility. Forages of earlier maturity are also thought to have higher protein content than those of later maturity. Therefore, grass consumed at earlier maturity is thought to produce more enteric CH_4 then grass at later maturity because this grass would have a higher protein content and lower lignin content. The partial breakdown of lignin in the rumen can also release *p*-coumaric acid and *p*-hydroxybenzaldehyde, which are toxic to rumen microbes. Jung (1985) found that *p*-hydroxybenzaldehyde decreased cellulose and hemicellulose in vitro dry matter disappearance (IVDMD), and *p*-coumaric acid decreased IVDMD after incubation for 48 hr. Chesson et al. (1982) found that populations of rumen bacteria differed in their tolerance of phenolics. Later maturity forages with more lignin are likely to result in greater release of these metabolites which could have a toxic effect on the rumen microbes (Jung, 1985).

Giger-Reverdin et al. (2003) found that supplementation with unsaturated fatty acids in the diet caused a decrease in CH_4 emissions. This is thought to be because polyunsaturated fatty acids perhaps provide another H acceptor or because they are toxic to the rumen microbes (Henderson, 1973). However, this also had other negative consequences such as increased feed refusals and decreased cellulose digestion. Foley et al. (2009) also found that supplementation of D-L Malic acid decreased CH_4 emissions by about 9% per unit DMI but this also caused animals to consume less feed. Feeding high levels of fat also reduces CH_4 production. Dong et al. (1997) investigated the effects of canola oil, cod liver oil or coconut oil supplementation on CH_4 production in an artificial rumen. All of the oils especially canola oil decreased CH_4 production and methanogenic bacteria populations regardless of diet. Beauchemin et al. (2007) investigated the effects of tallow, sunflower oil and whole sunflower seeds as fat sources on methane emissions and found that adding about 3% lipid to high forage diets in the form of saturated and unsaturated fatty acids decreased CH_4 emissions. However, out of all of these sunflower oil appeared to be the best to use because it had the least effect on fiber digestibility.

Feeding high levels of grain in the diet also helps to lower CH_4 levels in the rumen due to increased propionate to acetate ratio as found by Mc Geough et al. (2010). However, high levels of grain can also decrease ruminal pH, thus potentially resulting in an acidotic state. McGinn et al. (2009) investigated the effects of feeding corn distiller's grain on CH_4 production and while distiller's grain did reduce CH_4

emissions, there may be some effects of higher N and ammonia in manure on nitrous oxide emissions. Feed processing also affects ruminal CH_4 production because more finely ground feeds and pelleted feeds generally decrease CH_4 production.

Measuring CH_4 Production

Over the years there has been an abundance of research done on ways to measure both enteric and environmental CH_4 production. Researchers have come up with ways to measure CH_4 production in live animals. One more current way to measure enteric CH_4 production in cattle is through the use of the sulphur hexafluoride tracer gas technique (Boadi and Wittenburg, 2002; Omniski et al., 2006). To measure CH_4 produced by the animal, a tracer source is placed in the rumen allowing CH_4 to be directly measured from samples gases collected through the mouth in a stainless steel Mercury collection canister with filter. This allows gases to be collected continuously over a 24 hour period. The collection canisters are typically suspended by a neck piece attached to a halter apparatus. Collected gases are then run through gas chromatography with a Molecular Sieve 0.5 mm and Poropak column QS for SF_6 and CH_4 respectively. Methane is identified and quantified by peak area and retention time. However, some limitations of this method are that there is high variation and it does not account for CH_4 produced during hind gut fermentation. To account for potential noise in measurements, canisters are also placed in the environment to monitor ambient gas levels.

Another way to measure CH_4 produced by individual animals is through enclosed chambers, typically head boxes or ventilation hoods. To minimize contamination, these collection chambers must be well sealed with a slight negative pressure to avoid outside contamination. The chambers are equipped with an infrared CH_4 sensor and CO_2 sensor, which measure gas concentrations in air flow. These chambers are designed to measure basal CH_4 production. This method measures CH_4 production at basal metabolism (McGinn et al., 2004).

Nutritionists have also worked at developing equations to help predict CH_4 production. Several of these equations have been developed to predict CH_4 production from VFA ratios, carbohydrate fractions, H_2 balance, and N and phosphorous utilization. According to Wolin (1960), the amount of acetate (a), propionate (p) and butyrate (b) can be determined from the moles of CO_2 and CH_4 produced. By using the equation $CH_4 = a + 2b - CO_2$, CH_4 can be determined by the VFA profile. However, there have been much debate over the accuracy of these equations and Eun et al. (2004) found that this equation underestimated CH_4 production when compared with gas chromatography. This could be because stoichiometric equations do not consider microbial cells as end products of fermentation, and these cells can impact levels of CH_4 production. The amount of substrate available for microbial biomass can vary without changes in VFA proportions (Eun et al., 2004).

Kebrab et al. (2008) compared the accuracy of the COWPOLL, MOLLY, IPCC and Moe and Tyrrell models. These models were all designed to estimate CH_4 production. The models COWPOLL and MOLLY are dynamic mechanistic models that attempt to simulate CH_4 emissions based on fermentation patterns in the rumen. Moe and Tyrell and IPCC are statistical models that relate nutrient intake to CH_4 output (Kebreab et al., 2008). MOLLY uses VFA stoichiometry similarly to the equation demonstrated by Wolin (1960) but takes H_2 partitioning into account. Moe and Tyrell (1979) relates intake of carbohydrate fractions to CH_4 production: CH_4(MJ/d) = 0.341 +0.51non-soluble carbohydrate +1.74hemicellulose + 2.65cellulose. In comparing IPCC (IPCC, 2006), COWPOLL (Dijkstra et al., 1992;Kebreab et al., 2004), MOLLY (Baldwin, 1995; MOLLY, 2007), and Moe and Tyrell, COWPOLL appears to have the best accuracy and precision in predicting CH_4. However, a major disadvantage to these systems is that they provide only spot measurements of CH_4. Methane production is highly dependent on time after eating. In order to best understand the dynamics of CH_4 production in the rumen, continuous monitoring is needed.

Russomanno et al. (2012) have looked at feeding byproducts from human food to cattle and their relationship to greenhouse gas emissions. Russomanno et al. (2012) developed an equation to predict CH_4 from byproducts based on a previously existing model from Mills et al. (2003) to include byproduct emission estimation: $\%CH_4\ (BP_i) = [45.98-(45.98e^{-(-0.0011x\ \sum[starch/ADF]+\ 0.0445)\ x\sum MEI)}]$. BP_i refers to the starch, acid detergent fiber (ADF) and metabolizable energy intake (MEI) contained within the byproduct fed.

The equation uses compiled nutrient values for various by-products from the Cornell Net Carbohydrate and Protein System (CNCPS) to determine CH_4 emissions. The original equation developed by Mills et al. (2003) was CH_4 (MJ/d) = 7.30 + 13.13N(kg/d) + 2.04ADF(kg/d) +0.33Starch(kg/d). Mills et al. (2003) developed four different linear equations to measure CH_4 and these equations account for factors such as DMI, forage proportion, MEI, N intake (kg/d), ADF intake (kg/d) and starch intake (kg/d). However, these four developed significantly overestimated CH_4 production when evaluated against data collected in the United States, and the models were unable to match the low error of prediction seen in Moe and Tyrell.

CHAPTER TWO

METHANE PREDICTION BY NUTRIENT PROFILES IN RUMINAL CONTINUOUS CULTURES FED AN ALL FORAGE DIET OF BERMUDAGRASS OR ANNUAL RYEGRASS

ABSTRACT

Grazing intensive dairies (GiDs) in the southeast are evaluated for efficient milk production as well as environmental impact. The objective was to examine the ability of developed equations to predict methane (CH_4) in an all pasture-grazed diet, and determine the best predictors of CH_4 in grazing systems. In the first experiment, Tifton 85 bermudagrass (*Cynodon dactylon x Cynodon nlemfuencis)* was harvested at five dates regrowth (14 d, 21 d, 28 d, 35 d and 42 d) from plots at the University of Georgia in fall 2011, freeze-dried, and ground (2-mm sieve). In the second experiment Marshall annual ryegrass (*Lolium multiflorum)* was harvested at the same five days regrowth from plots at the University of Georgia in the winter 2013, freeze-dried and ground (2-mm sieve). For both experiments, thirty grams of harvested forage were fed daily to five separate dual-flow continuous fermenters equipped with a gas sensor system to measure CH_4 concentrations in headspace for three 7 d periods. Both experiments were arranged in a randomized block design with fermenter as block. Acetate: propionate and VFA proportions for bermudagrass were not different between days regrowth. For annual ryegrass, there were treatment, time and treatment*time interactions for most of the VFA as well as acetate: propionate so differences were unclear. Feeding 28 d

bermudagrass had higher (*P<0.001)* CH_4 production compared to all other treatments except for 35 d. In experiment 2, feeding annual ryegrass at 21 d of regrowth resulted in the highest (*P<0.0001)* CH_4 produced compared to all others. Both Mills et al (2003) and Moe and Tyrrell as cited by Ellis et al. (2007) over predicted CH_4 compared to measured values but the difference was greater for annual ryegrass. Methane production with time increased (*P < 0.001)* from 0800 to 1600 h for both experiments although there was a treatment x time interaction (*P<0.01)* in experiment 2 for annual ryegrass. In experiment 1, CH_4 expressed per g NDF-D bermudagrass had higher (*P<0.0001)* CH_4 production at 14 d compared to all others. Methane expressed per g DM apparently digested was also higher (*P<0.05)* at 14 d although this was similar to 28 d and 35 d. Expressing CH_4 on a per g NDF-D basis or per g apparently digested made minimal difference between annual ryegrasses. Digestibility is a function of CH_4 production but does not account for all differences between grass maturities so nutrient correlations and regressions were performed. For both experiments, starch (*P<0.0001)*, sugar (*P<0.05),* and hemicellulose (*P<0.05)* content were positively correlated with average daily CH_4, and NDF was positively correlated *(P*<0.05) with CH_4 when feeding bermudagrass and had a positive trend (*P<0.10)* when feeding annual ryegrass. Acid detergent lignin (ADL) and CP were negatively correlated (*P <0.001)* with CH_4 when feeding bermudagrass. The forward regressions for both experiments show that starch is the strongest predictor (*P<0.0001)* of CH_4 in for both grass types and ADL is also a common predictor (*P<0.01)*. Forward regressions also show that sugar was a significant

predictor (*P<0.001)* of CH_4 only in bermudagrass while hemicellulose was a significant predictor (*P<0.001)* of CH_4 only in annual ryegrass. Data suggest that forage starch content may be the best common predictor of rumen CH_4 production when feeding an all pasture diet but other contributing factors may vary between forage species.

INTRODUCTION

In the southeast region, there has been an increasing shift away from traditional confinement feeding dairy systems (TCDs) and towards grazing intensive dairies (GiDs). A GiD is a system in which cattle rely on grazing for the majority of their diet, spend more than 90% of their time on pasture, and deposit more than 90% of their waste on the soil surface directly (White et al., 2001). Typically cattle are grazed on a paddock and then moved to another paddock to allow uniform regrowth of the grass. Grazing intensive dairy systems are favorable in the southeast due to warmer weather and a longer grazing season. These systems can be more economically efficient due to reduced labor and operating costs even though milk production is usually lower since GiDs cannot optimize nutrients as well as confinement systems (Dartt et al., 1999). One common type of grass used in these GiDs is bermudagrass, a warm season grass that is productive in May thru October. It's ability to grow well on sandy soils and extreme drought tolerance makes bermudagrass a good grass type to include in GiDs. Bermudagrass is also tolerant of close continuous grazing and tends to grow best under these conditions (Ball et al., 2002). However, bermudagrass tends to lignify quickly and must be grazed aggresively to maximize milk production. Annual ryegrass is a popular

cool season grass found in GiDs. Its highest forage productivity is February through May. Annual ryegrass tolerates poorly drained soil and like bermudagrass, is also tolerant of close, continuous grazing (Ball et al., 2002)

These GiDs also have an effect on the environment. Increasing the amount of forage fed increases the acetate: propionate ratio in the rumen, which is thought to be correlated to increased ruminal CH_4 production (Van Kessel and Russell, 1996), thus potentially increasing the CH_4 in the environment. Dairy cattle are thought to produce approximately 120 L/d of CH_4. Agriculture is thought to contribute about 8% of the total greenhouse gas emissions in the US and is the second largest source of CH_4 (EPA, 2007). The increasing levels of CH_4 in the environment have been a rising concern since CH_4 is 21 times more potent in its ability to trap heat in the environment than CO_2 (Kebreab et al., 2008).

Equations have been developed to predict CH_4 based on the nutrient profile of the diet. However, many of these equations have been developed based on diets of forage and concentrate mixture instead of all forage. To determine the best predictors of CH_4 when feeding an all-forage diet, Tifton 85 bermudagrass or Marshall annual ryegrass of varying days regrowth were fed to an artificial ruminal digestor equipped with a gas sensor system to monitor CH_4 production over time. Relationships between CH_4 produced and nutrients in forages were analyzed, and comparisons between predicted and measured CH_4 were made.

MATERIALS AND METHODS

Experiment 1

Treatments consisted of Tifton 85 bermudagrass harvested at five different days regrowth (14 d, 21 d, 28 d, 35 d, and 42 d) with forage of a different regrowth period fed to each fermenter for three 7 d periods. Bermudagrass was grown in pre-existing Tifton 85 field plots at the University of Georgia plant science farm (Watkinsville, GA). On September 1st, 2011, plants were mowed to a height of 5 cm, fertilized with 100 kg/ha N and then harvested at 14 d, 21 d, 28 d 35 d, and 42 d regrowth after mowing. All bermudagrasses were in the vegetative state at harvest regardless of days regrowth. Harvested plants were then frozen, lyophilized, and ground through 2 mm screen in a Wiley Mill. A total of 30 g fresh matter of the diet was inoculated in the fermenter, and added daily in two equal amounts at 0800 and 1600 h.

Experiment 2

Treatments consisted of Marshall annual ryegrass harvested at five different days regrowth (14 d, 21 d, 28 d, 35 d, and 42 d) with forage of a different regrowth period fed to each fermenter for three 7 d periods. Annual ryegrass was planted at the University of Georgia J. Phil Campbell Research and Education Center (Watkinsville, GA) on October 7th 2012. Plants were fertilized with 50 kg/ha N at planting, then mowed to a height of 5 cm on January 3rd 2013 and fertilized again with 80 kg/ha N. Annual ryegrasses were harvested at 14 d, 21 d , 28 d, 35 d and 42 d regrowth after mowing. All

ryegrasses were in the vegetative state at harvest regardless of days regrowth. Harvested plants were then frozen, lyophilized, and ground through 2 mm screen in a Wiley Mill. A total of 30 g fresh matter of the diet was inoculated in the fermenter, and added daily in two equal amounts at 0800 and 1600 h.

Continuous Culture Conditions

Whole rumen contents were taken from a ruminally-fistulated Holstein cow being fed a 50% forage/ 50% concentrate diet, and filtered through double-layer cheesecloth prior to incubation in the fermenters. All surgical and animal care protocols were approved by the Clemson University and Animal Care and Use Committee. Rumen fluid was strained through 2 layers of cheesecloth, and approximately 20 minutes after collection and straining, strained rumen fluid was combined in a 1:1 dilution with prepared buffer solution as described by Slyter et al. (1966). Approximately 800 mL of this rumen fluid and buffer mixture was transferred to each of the dual-flow fermenters. Continuous cultures in this study were an all glass, closed system with independent flow of liquid and particulate matter (Appendix A). This continuous culture design was modified from the design described by Teather and Sauer (1988). The modification included an overflow sidearm angle of 45° to facilitate emptying of overflow, a faster stirring rate of 60 rpm that still allowed for stratification of feed particles into an upper fiber mat, middle liquid portion and lower dense mat, and a feeding rate of 30 g/d. Rubber seals and continuous flow of CO_2 (20 mL/min) help to maintain an anaerobic environment and positive pressure in the culture. Artificial saliva (Slyter et al., 1966) was

delivered using a precision pump set at a flow rate of 90 mL/hr to maintain a 10-12% liquid dilution rate in the culture. Each day buffer was adjusted using 6N NaOH or 3N HCl so that the AM pH was maintained between 6.5-6.6 while the PM pH was allowed to fluctuate based on diet fed. The temperature was kept at 39°C by a circulating heated water bath. Fermenters were run for 7 days with the first 4 days for adaptation to the diet and the last 3 days for sampling.

Culture pH was monitored daily by taking pH readings (Hanna Instruments, Inc., Woonsocket, RI.) before each feeding. Culture contents were thoroughly mixed at approximately 155 RPM prior to taking pH readings or samples. A 4-mL sample of culture contents were taken on d 7 of each period at 0 (before the 0800 h feeding), 2, and 4 h after feeding for analysis of VFA. Overflow was measured daily but was collected from each fermenter in a 2 L Erlenmeyer flask kept in a covered ice bath on d 5, 6 and 7 of each period. Total volume was recorded, and a 20% sample of the overflow was combined between sampling times and immediately frozen. Frozen samples were later thawed and lyophilized. Overflow contents were mixed continuously with a magnetic stir bar during all samplings.

Chemical Analysis

Culture VFA samples were pipetted into polycarbonate tubes containing 1-mL of 25% (w/w) metaphosphoric acid, centrifuged at 31,600 x *g* for 20 minutes at 4° C, and 1 mL of supernatant was collected and combined with 100µl 2-ethylbutyric acid (86

μmol/100μL) as an internal standard. Samples were analyzed by gas chromatography (GC) with flame ionization (FID) detector on a Zebron ZB-FFAP 30 m x 0.25 mm x 0.25μm column (Phenomenex, Torrance, CA). Ten mL of sample was taken each morning on days 5, 6, and 7, and centrifuged at 31,600 x *g* for 20 minutes with the pellet used for DM analysis (100 °C). Forage and dried overflow samples were ground in a centrifugal mill through a 0.5 mm sieve prior to analyses.

Dried forage samples were analyzed for nutrient content by Cumberland Valley Analytical Laboratories (Maugansville, MD). Analyses included determination of crude protein (CP) (AOAC, 2000), soluble protein (Krishnamoorthy et al., 1982), rumen degradable protein (Krishnamoorthy et al., 1983), acid detergent lignin (ADL) (Goering and Van Soest, 1970), lignin/NDF ratio, sugar (Dubois et al., 1956), and starch(Bach Knudsen, 1997). Forage samples in addition to culture samples were also analyzed for neutral detergent fiber (NDF) using α-amylase as suggested by Van Soest et al. (1991) and acid detergent fiber (ADF) as described by AOAC (1990 No. 973.18). Grams of DM apparently digested were measured as DM in minus DM out of the culture, and NFD-D was measured as NDF in- NDF out.

CH_4 Analysis

Methane concentrations were taken in continuous cultures through the use of a custom built, gas sensor system. The custom-built system (Appendix B) involves the use of infrared CH_4 and CO_2 sensors (Edinburgh Instruments, OEM Gas Sensors, Great

Britain) and an O_2 infrared sensor (KE Series, Figaro USA Inc, Arlington Heights, IL) to measure gas concentration in a 1 L headspace sample. The three sensors are calibrated to 100% and mounted on a stainless steel box with an air pump, and a signal processor connected to a computer to record data. Sample is pulled through silica tubing of 3.18 mm I.D x 6.35 mm O.D (Dow Corning Corporation, Midland MI) through one of eight two-way pinch valves (Valcor Scientific, Springfield, NJ) to allow sampling of one culture at a time. A data acquisitioner is mounted on the box, and each of the sensors gives out a voltage reading (0-5 V) measured by the data acquisitioner and amplified by 20 to get the gas percentage. Readings are taken continuously on each fermenter allowing changes in gas concentrations to be monitored over time. The sensors were calibrated at the beginning of each trial in order to ensure accuracy with the O_2 sensor calibrated by the manufacturer and the CO_2 and CH_4 sensors calibrated with gas mixtures of known composition (Appendix C). Methane was recorded every hour between 0800 and 1600 h on days 5, 6 and 7. Methane percent was then converted to mmol/d by using the total gas flow rate per day (20 mL/min) multiplied by the percent CH_4 measured, divided by the gas constant 22.4 mol/L, and divided by 1000 to get CH_4 in mmol/d.

In addition to measuring CH_4 two equations were used to predict CH_4 based on the nutrient profiles of the diets. The first equation was developed by Mills et al. (2003) and was modified to the following:

Experiment 1: CH_4 (MJ/d) = 0.0057 + 13.13N(kg/d) + 2.04ADF(kg/d) +0.33Starch(kg/d)

Experiment 2: CH_4(MJ/d) = 0.0052 +13.13N(kg/d) +2.04ADF(kg/d) +0.33Starch(kg/d)

The second equation was developed by Moe and Tyrrell (1979), but cited by Ellis et al., (2007) was modified to the following:

Experiment 1: CH_4 (MJ/d) =0.0057 +0.511NSC(kg/d) + 1.74HC(kg/d) + 2.652C(kg/d)

Experiment 2: CH_4 (MJ/d) = 0.0052 +0.511NSC(kg/d) + 1.74HC(kg/d) + 2.652C(kg/d)

In these equations NSC= nonsoluble carbohydrates, HC=hemicellulose, C=cellulose, N=nitrogen, and ADF=acid detergent fiber. For both equations, the original intercept was dropped out and replaced by the average CH_4 readings at 0800 h for each experiment to modify the equation for continuous culture use. Methane in (MJ/d) was also converted to mmol/d by dividing values by 0.891 since there are 0.891 MJ/mol, and then multiplied by 1000 to convert mol to mmol.

Statistical Analysis

Data were analyzed in SAS version 9.2. CH_4 production and VFA composition was analyzed by PROC GLIMMIX procedure in SAS 9.2. Both CH_4 and VFA data were analyzed with treatment, time and their interaction as fixed effects. Random effects for CH_4 production included day, period and their interaction while random effects for VFA included period, fermenter, and diet*period interaction. Correlation between grass nutrient content and CH_4 production were performed using the PROC CORR feature in SAS 9.2. A forward stepwise regression was performed to further examine nutrient

effects on CH_4 production using PROC REG. The nutrients correlated with CH_4, and entered into the regression were ADF, NDF, ADL, sugar, starch, cellulose, hemicellulose, and CP. Along with the regressions, co-linearity among nutrients was checked for using the VIF command in SAS, and a VIF greater than 10 was considered an indication of co-linearity. Throughout, a *P*-value of < 0.05 was considered to be significant unless otherwise noted and trends were also considered at a value of *P<0.10.*

RESULTS

Experiment 1

Ruminal continuous cultures are used as a model of the rumen environment and thus should reasonably replicate in vivo fermentation conditions. Throughout the experiment, CO_2 and O_2 percentages in the cultures averaged 77% and 0.5% respectively prior to AM feedings indicating a mostly anaerobic environment in the cultures. Average VFA proportions (mol per 100 mol*)* for experiment 1 are reported in table 1. Overall, there was no effect of days regrowth on proportions of VFA. There was also no effect of time aside from a decrease in the proportion of isobutyrate (Table 1) between 2 and 4 h (*P <0.05)* indicating that fermentation was similar for all grasses. Acetate: propionate ratio ranged from 4.4-4.9 when averaged over times and there was no significant difference between days regrowth. There was no significant difference of culture pH with treatment but there was an overall time effect of pH being higher (*P<0.01)* at time 0 than 2 and 4 h.

Nutrient composition of Tifton 85 bermudagrass is shown in table 2. All nutrients were expressed in single samples, therefore statistics were not run. There was a 28.5% difference in CP between the lowest value at 42 d and the highest value at 14 d. There was a difference of 40% between the lowest at 35 d and the highest at 14 d for ADL. The ADL/NDF ratio following a similar pattern except the difference between 35 d and

14 d was 65.3%. There were differences of 16.5% and 3.7% in soluble protein and rumen degradable protein content respectively with 35 d having the lowest soluble and rumen degradable contents and 42 d having the highest. Starch and sugar differences were 37.8% and 28.1% respectively with 14 d having the lowest sugar content and 21 d having the lowest starch content, and 28 d having the highest content of both sugar and starch. Finally, there was a 17.7% difference in NDF content between the lowest and highest (14 d and 35 d respectively), and a 9.7% difference in ADF between the grasses with the lowest (28 d) and highest (35 d) ADF content.

Data for CH_4 production by days regrowth and time of day (0800-1600 h) are shown in figure 1. For days regrowth, there was a linear increase (*P<0.001)* in CH_4 production between 0900 h and 1200 h with the lowest values at 0800 and 0900 h immediately before and after feeding. Methane production peaked at 1300 h, and remained steady. To compare measured to predicted CH_4, values were averaged over times 1300-1600 h (table 3) to depict maximum values of CH_4 production. Feeding bermudagrass at 28 d had the highest (*P<0.001)* maximum CH_4 compared to others except for 35 d. Maximum CH_4 production for bermudagrass at 14 d, 21 d and 42 d were all similar. The maximum CH_4 values for bermudagrass in this study were lower than those predicted by equations developed by Mills et al. (2003) or Moe and Tyrrell. The CH_4 values predicted by the Mills et al. equation (35.32-38.40 mmol/d) were closer to measured values (19.07-32.13 mmol/d) than those predicted by Moe and Tyrrell (39.52-47.35 mmol/d) even though both equations over predicted CH_4.

Methane expressed per g NDF-D (table 4) was highest (*P<0.0001)* at 14 d compared to all others. Methane expressed per g DM apparently digested (table 4) was higher (*P<0.05)* at 14 d than 21 d and 42 d but was similar to 28 d and 35 d. Expressing CH_4 values as a function of DM or NDF digestibility did not account for all treatment differences. Therefore, correlations with CH_4 and nutrients were analyzed.

Bermudagrass nutrients that were separately correlated with rumen CH_4 production are displayed in table 5. Sugar and starch were the most positively correlated (*P<0.0001)* with rumen CH_4 production while ADL and CP were the most negatively correlated (*P<0.001).* In addition, hemicellulose and NDF were also positively correlated (*P<0.05)* with rumen CH_4 production. Although there was significant correlation with several different nutrients, the three nutrients that met the 0.10 significance level to be included in the forward stepwise regression, and had VIF <10 (table 6) were starch (*P<0.001)*, sugar (*P<0.05),* and ADL (*P <0.10).* Although these three nutrients were the only ones included in the regression, ADF also met the 0.10 significance level to be included but due to co-linearity among nutrients (VIF >10), it could not be included in the model.

In running a regression to look the effects various nutrients on CH_4 production, one possible problem that can occur is co-linearity among the nutrients. This co-linearity can cause variance inflation and inaccurate regression coefficient estimates. This is especially true when trying to include multiple overlapping fiber components (such as

lignin, NDF and ADF) since these fiber components are typically very closely correlated with each other. One method to check for co-linearity is to include the variance inflation factor (VIF) option in SAS when estimating the regression model with PROC REG. A VIF over 10 for any of the nutrients is a sign of strong co-linearity among the nutrients. The VIF value was often over 10 in the regression models that included NDF, ADF and ADL together and therefore models including these nutrients together were not considered. Including ADL only in a regression model with starch and sugar resulted in a high R-square values without effects of co-linearity.

Experiment 2

Average VFA proportions (mol per100 mol) for annual ryegrass are shown in table 7. Overall, there was a treatment (*P<0.05)* and time effect (*P<0.05)* for all VFAs but also treatment*time interactions for all VFA except for valerate. For valerate, annual ryegrass at 14 d regrowth had a higher (*P<0.05)* proportion than 21 d or 35 d but was similar to 28 d and 42 d. Valerate also decreased (*P<0.0001)* with time between 0 h and 2 h and then increased again between 2 h and 4 h. Proportions of acetate stayed the same between 0 h and 4 h except for 14 d and 42 d which decreased (*P<0.05).* Acetate proportions between 2 h and 4 h were similar for all annual ryegrasses except for 28 d and 35 d in which proportions increased (*P<0.05).* Propionate proportions increased *(P<0.0001)* between 0 h and 4 h for 14 d and 42 d, were similar for 21 d and 28 d, and decreased for 35 d. Propionate proportions were similar between 2 h and 4 h for all

annual ryegrasses except for an increase (*P<0.0001)* with 42 d between those times. Annual ryegrasses 21 d and 35 d increased (*P<0.001)* in isobutyrate proportions between 0 h and 4 h, while proportions did not change for 28 d and 42 d, and decreased (*P<0.001)* for 14 d. Decreases in isobutyrate between 2 h and 4 h were also seen for all annual ryegrasses except for 21 d, which stayed the same, and 35 d which increased. All annual ryegrasses had similar butyrate proportions between 0 h and 4 h except for 28 d and 42 d which increased (*P <0.05).* Butyrate proportions between 2 h and 4 h were similar except for decreases with 14 d and 28 d. Isovalerate proportions were similar between 0 h and 4 h for all treatments except proportions decreased with 14 d and increased with 35 d *(P<0.01).* Acetate: propionate ratios decreased (*P<0.001)* between 0 h and 4 h for 14 d and 42 d but increased for 35 d and stayed the same for 21 d and 28 d. Ratios were similar for all annual ryegrasses between 2 h and 4 h except for decreases with 28 d and 42 d. All pH values were similar between 0 h and 4 h except for decreases (*P<0.05)* with 14 d and 42 d. As a result, effects of treatment on VFA were not clearly defined since they varied a great deal with time.

The nutrient profile of Marshall annual ryegrass is shown in table 8. All nutrients were expressed in single samples, therefore statistics were not run. Crude protein content increased more than 110% between the lowest content at 14 d ryegrass and highest content at 28 d. Acid detergent lignin had a 75% change between the lowest and highest values which were seen in 28 d and 35 d ryegrasses while the ADL/NDF followed the same pattern but with a 59.4% change. Soluble protein and rumen degradable

protein had 51.9% and 11.6% changes respectively with the lowest contents seen in 35 d grass and the highest in 28 d. Finally, amount of sugar in 14 d ryegrass was more than 4 times the amount in the 35 d ryegrass with a difference of 431%, and the amount of starch in 21 d ryegrass was one and a half times the amount in 35 d ryegrass with a 150% change.

Data for CH_4 production for each days regrowth by time of day (0800-1600 h) is shown in figure 2. There was a linear increase (*P <0.001)* in CH_4 with time from 1000 h-1400 h. The lowest production was at 0800 h, 0900 h and 1000 h right before and after feeding. Methane production peaked around 1400 h and stayed steady. There was also a treatment x time interaction (*P<0.01)* since the five annual ryegrasses of different days regrowth were the same prior to 1100 h but differed at later times.

To compare CH_4 as predicted by the Mills et al (2003) and Moe and Tyrrell equations to values measured, numbers were averaged over 1300-1600 h (table 3). Feeding annual ryegrass at 21 d regrowth resulted in the highest (*P <0.0001)* CH_4 compared to all other treatments, while feeding annual ryegrass at 35 d resulted in the lowest (*P<0.0001)* CH_4 produced compared to all others. Similar to experiment 1, both Mills et al. (2003) and Moe and Tyrrell overestimated CH_4 production compared to measured values although Moe and Tyrrell yielded slightly closer values (27.78-35.33 mmol/d) to measured CH_4 (8.14-17.21 mmol/d) compared to Mills et al (34.78-42.31 mmol/d). Differences among treatments were the same when results were expressed

per g DM apparently digested or g NDF-D except 28 d and 14 d were similar when expressed per g NDF-D (*P<0.0001)* (table 4). Like experiment 1, digestibility did not account for all differences between grasses so correlations of CH_4 between nutrients were explored.

Methane correlated with forage nutrients (table 5) shows starch (*P <0.0001)* and hemicellulose (*P<0.001)* as well as sugar (*P<0.05)* to be positively correlated with rumen CH_4 production. Neutral detergent fiber had a trend towards being positively correlated *(P<0.10)* as well. Although there were several nutrients correlated with CH_4 production in annual ryegrass, the three nutrients that met the 0.10 significance level, and had a VIF <10 to be included in the forward stepwise regression were starch (*P<0.0001)*, hemicellulose (*P <0.0001),* and ADL (*P<0.05)* (table 9).

DISCUSSION

Although there were no significant differences between the VFA profile between forage days regrowth for bermudagrass, Hindrichsen et al. (2004) found CH_4 to be correlated with butyrate and propionate production. However, Doane et al. (1997) found that VFA production and NDF disappearance did not differ between mature and immature forages. The acetate: propionate ratios in experiment 1 were similar to those found by Pordomingo et al. (1991), Galloway et al. (1993a), and Mathis et al. (2000) indicating that this bermudagrass was fermented similarly to grasses in other studies. However, although higher acetate to propionate ratio has been thought to be associated with higher CH_4 production (Johnson and Johnson, 1995), Daone et al. (1997) found the acetate: propionate ratio to be a poor predictor of CH_4 production. It is likely that microbial yield and growth rate are also important in determining gas production per mmol of VFA (Krishnamoorthy et al., 1991, Van Soest, 1994). Although the interactions made it difficult to determine treatment effect on VFA in annual ryegrass, the acetate to propionate proportion ratios for each annual ryegrass averaged over time ranged from 2.1-2.9 compared with the 4.4-4.9 ratio range seen for bermudagrasses. The higher proportions of propionate and lower proportions of acetate observed when feeding annual ryegrass may be a contributing factor to the lower amounts of CH_4 produced. While acetate production increases the amount of H_2, propionate production utilizes H_2 thus reducing the amount of free H_2 for methanogenesis (Johnson and Johnson, 1995; McGinn et al., 2004).

The increase in CH_4 production after feeding is likely due to increased substrates for methanogenesis. Bacteria, protozoa and fungi ferment feed to VFA, which also produces the CO_2, H_2, and acetate needed to produce CH_4. At 0800 h, the cultures have been not been fed for 16 hours thus limiting feed available for fermentation, and CH_4 levels. The CH_4 production pattern with time seen in this study may not be the exact pattern seen in a grazing system since cattle are allowed unlimited access to feed. However, cattle typically spend 8 hours a day ruminating which is about equivalent to the time between feedings. Although the CH_4 values obtained when feeding bermudagrass in experiment 1 were close to predicted values by Mills et al. (2003), CH_4 values measured when feeding annual ryegrass were considerably lower than both predicted values and those in experiment 1. Annual ryegrass typically has a higher soluble sugar content compared to bermudagrass, and based on visual observations during experiment 2, does not result in a thick fiber "mat." A thick fiber mat helps to foster growth and attachment of rumen microbes, and increase rumen activity which then increases substrates for methanogenesis (Welch, 1982). Lack of a thick fiber mat could result in less microbial growth which in turn could also partially explain the lower amounts of CH_4 seen with annual ryegrass compared to bermudagrass.

Although feeding annual ryegrass resulted in lower CH_4 production compared to bermudagrass in these experiments, this may not necessarily be true in a GiD where cattle are allowed to graze ad libitum instead of only being fed twice per day. The rapid digestibility of annual ryegrass would likely cause cattle to graze more, resulting in

increased substrates for methanogenesis. Furthermore, farms will typically feed wheat straw along with annual ryegrass to slow rate of passage so that nutrient absorption can be optimized which may also result in more CH_4 produced. Feeding wheat straw could also potentially increase the fiber mat which would increase attachment for microbes.

Eun et al. (2004) also measured CH_4 production in dual-flow continuous cultures via gas chromatography and found that when cultures were fed a high forage diet at a 12.5% dilution rate, CH_4 production was found to range from 20.10-29.10 mmol/d. The CH_4 readings measured in experiment 1 ranged between 19.07-32.13 mmol/d and the readings from experiment 2 ranged from 8.14-17.21 mmol/d. The readings taken by Eun et al. were taken approximately two hours after feeding while the readings in this study were averaged over 1300 h-1600 h to give maximum CH_4. Forages are fermented more slowly than concentrate diets or diets that are a mixture of both. The slight variation in CH_4 numbers between the values found by Eun et al. (2004) and the values found in these two studies may be accounted for by forage type and amount of forage fed. Eun et al. (2004) fed alfalfa instead of bermudagrass or annual ryegrass and the feeding rate was only 12.8-13.0 g/d compared to 30 g/d in this study.

In addition to research done on dietary factors that affect CH_4 production, much effort has been directed at developing equations to predict CH_4. These equations based predictions of ruminal CH_4 on VFA fermentation profiles, intake, digestibility or nutrient profiles in the diet fed (Blummel, 1997; Mills et al., 2003, Moe and Tyrrell, as cited by

Ellis et al. (2007) as predictors of CH_4. These prediction equations however, were developed using data from feedlot cattle or cattle fed TMR instead of pasture, which can better optimize production and nutrient intake. As a result, it is questionable as to whether these equations are accurate predictors of CH_4 for cattle in grazing systems. In a grazing system, it is difficult for the farmer to measure VFA fermentation profiles or digestibility so getting a forage sample analyzed and using the nutrient profile to predict CH_4 would be the easiest method. The two equations used in these studies to predict CH_4 each use slightly different nutrient components. For the purposes of these studies, the intercepts for each equation were modified to fit lower feeding rates for continuous fermenters. Both equations had intercepts that represent CH_4 production in a cow before feeding. By dropping this intercept and replacing it with the CH_4 readings taken at 0800 h before feeding, these equations were adapted for continuous culture use.

Both Mills et al.(2003) and Moe and Tyrrell use non-structural carbohydrate levels in the diet to predict CH_4, and many equations such as these use regressions to determine nutrients to be included in the model. In experiments 1 and 2, CH_4 production was found to be significantly correlated with several nutrients including sugar, starch, HC, and ADL. However, PROC CORR only correlates single nutrients and does not take into effect the interactions of nutrients. Therefore a forward stepwise regression was used determine if multiple nutrients are a better CH_4 predictor. Hindrichsen et al. (2004) observed the effects of different carbohydrate sources with

differing sugar contents on rumen CH_4 production in vitro and found that CH_4 release (mmol/g of organic degraded matter) increased with increasing diet sugar content with various carbohydrate sources.

A similar pattern was also seen in CH_4 emissions from cattle (Hindrichsen et al., 2005). In this case, increased sugar and starch content was positively correlated with increased CH_4 production even though others have found that increased starch content was found to be associated with decreased CH_4 production (Hassenat et al., 2013; Lovett et al., 2003; Harper et al., 1999). However, it is important to note that this pattern in CH_4 emission may also depend on ruminal pH. Generally feeding a diet with a high sugar or starch content that is rapidly fermentable tends to drop ruminal pH below 6.0, thus explaining the drop in CH_4 typically seen when feeding high levels of concentrate. Methanogens and protozoa are highly sensitive to low pH, which could compromise CH_4 production. Fermentation of forages like bermudagrass and annual ryegrass cause less production of acid in the rumen thus resulting a higher ruminal pH. A pH between 6.0-7.0 is ideal for cellulolytic activity. Although Hindrichson et al. (2004) did not feed an all-forage diet; the experiment used a continuous culture setup in which pH was intentionally kept at a certain range instead of being allowed to fluctuate. Ellis et al. (2012) observed the effect of high sugar grasses on using a simulation system. Simulation results showed that biggest increases in CH_4 production occurred when water soluble carbohydrate (WSC) content increased at the expense of CP or NDF

content in grasses although the effect was lower when WSC increased at the expense of NDF verses CP. This supports data in experiment 1, in which CH_4 production was positively correlated with sugar content but negatively correlated with CP.

Ellis et al. (2012) also found that CH_4 emissions increased when WSC increased at the expense of NDF but NDF content and NDF digestibility may change in opposite directions. Increases in NDF-D as well as apparent DM digestibility in experiment 1 accounted for some of the differences in CH_4 production between bermudagrasses likely due to increased available substrates. However, apparent DM digestibility did not account for any differences between ryegrass maturity dates and NDF-D only accounted for a small difference between 14 d and 28 d. In this study, only apparent DM digestibility was reported, which does not include overflow microbial matter. Furthermore, there were several interactions between ryegrass maturity dates making differences due to treatment difficult to see. Forage NDF was positively correlated in experiment 1 and had a positive trend in experiment 2 even though it did not make the significance level to be included in the regression. Ellis et al. (2007) found that NDF was positively correlated with CH_4 production when expressed as kg per day. Increased fiber in the diet is thought to increase rumen fermentation, slow down passage rate and increase acetate: propionate ratio (Boadi et al., 2004; Benchaar et al., 2001)

According to Moe and Tyrrell (1979), hemicellulose concentrations in the diet are thought to be positively correlated with CH_4, which was true with both grass types

even though it was only included in the ryegrass regression. Hemicelluloses have been thought to stimulate propionate production (Marounek et al., 1985) which would link them with decreasing methane production. In a study by Czerkawski and Breckenridge (1969), hemicelluloses had no effect on methanogenesis. This unclear relationship may be due to differing chemical compositions of different feeds.

In both studies, acid detergent lignin (ADL) was negatively correlated with CH_4 production although this correlation was significant with bermudagrass but not with annual ryegrass. This may be because bermudagrass tends to lignify more quickly, and has a higher percent ADL than ryegrass. Acid detergent lignin was also included in the stepwise regression as negatively impacting CH_4 production for bermudagrass but positive for ryegrass even though the correlation was slightly negative. Ellis et al. (2007) found that when lignin was included in a complex regression equation its overall effect on CH_4 was negative. It is generally thought that lignin tends to increase with forage maturity (Van Soest et al., 1994). However, since acid detergent lignin is not necessarily "true lignin" and includes other compounds such as tannins, this may explain why the lignin to forage days regrowth relationship was not linear.

CONCLUSIONS

Methane production in ruminal continuous cultures fed Tifton 85 bermudagrass or Marshall annual ryegrass was best predicted by forage starch content. These studies show that starches are likely to have a higher CH_4 producing effect under conditions of high ruminal pH, which is typical in diets containing a high proportion of forage. Equations developed to predict CH_4 consistently overestimated the amount of CH_4 even though these equations do use both fibrous and non-fibrous carbohydrates to predict CH_4. This may be due to the equations being developed from cattle consuming a TMR or feedlot diet, which can optimize nutrient intake better than a grazing diet but may result in a lower ruminal pH. Varying chemical compositions and nutrient interactions in different grass types are likely the reason that a regression may be more effective than correlations to identify key nutrients and their effect on CH_4 production. When feeding an all pasture-grazed diet, starch content is the best common predictor of CH_4 but other effective predictors may exist or may vary between grass species.

Chapter 2 Tables

Table 1. Experiment 1 volatile fatty acids (mol/100 mol). The fermentation profile when feeding Tifton 85 bermudagrass at 14 d, 21 d, 28 d, 35 d, and 42 d regrowth at 0, 2 and 4 h after feeding. Means were calculated by LS means and data was reported significant if *P<0.05.*

	Bermudagrass						*P-value*		
VFA	14 d	21 d	28 d	35 d	42 d	SE	Diet	Time	Diet*time
Acetate						2.50	0.51	0.33	0.50
0 h	74.8	73.5	77.4	69.3	73.3				
2 h	73.0	73.7	71.5	70.4	70.5				
4 h	77.1	72.5	71.4	72.1	74.5				
Propionate						1.36	0.77	0.16	0.64
0 h	16.7	16.7	14.2	17.0	16.2				
2 h	17.6	16.3	16.0	16.6	16.4				
4 h	15.5	16.3	15.6	15.7	14.8				
Isobutyrate						0.09	0.50	0.02	0.14
0 h	0.6	0.4	0.5	0.6	0.7				
2 h	0.6	0.4	0.7	0.7	0.7				
4 h	0.3	0.4	0.6	0.5	0.5				
Butyrate						0.81	0.24	0.12	0.57
0 h	6.0	7.1	6.0	9.4	7.0				
2 h	6.9	7.7	8.9	9.2	9.2				
4 h	5.7	8.5	9.3	8.9	7.7				
Isovalerate						0.26	0.14	0.24	0.25
0 h	1.0	1.1	1.1	2.6	1.8				
2 h	1.0	0.8	1.6	1.8	2.1				
4 h	0.5	1.0	1.7	1.6	1.4				
Valerate						0.20	0.48	0.72	0.69
0 h	1.0	1.1	0.8	1.5	1.1				
2 h	1.1	1.1	1.3	1.4	1.1				
4 h	0.8	1.3	1.3	1.3	1.0				
Acetate:Propionate						0.55	0.75	0.16	0.53
0 h	4.6	4.6	5.6	4.1	4.6				
2 h	4.3	4.6	4.6	4.3	4.4				
4 h	5.2	4.6	4.7	4.7	5.1				
pH									
0 h	6.7	6.4	6.4	6.5	6.5	0.09	0.10	0.01	0.97
2 h	6.6	6.3	6.3	6.4	6.3				
4 h	6.6	6.4	6.3	6.5	6.4				

Table 2. Experiment 1 nutrient content of Tifton 85 bermudagrass (DM basis unless otherwise stated) for grasses of 14 d, 21 d, 28 d, 35 d, and 42 d regrowth. The analyses were run as single samples without statistics. All analyses were done at Cumberland Valley Analytical Laboratories in Hagerstown, MD.

Item	Bermudagrass by Days Regrowth					
	14-d	**21-d**	**28-d**	**35-d**	**42-d**	**%Change[1]**
DM, %	91.6	92.6	94.9	94.9	93.8	3.6%
CP, %	21.2	19.0	16.1	15.4	15.3	28.5%
Soluble Protein, % CP	31.1	33.8	34.2	29.7	34.6	16.5%
Rumen Degradable Protein, % CP	65.5	66.9	67.1	64.9	67.3	3.7%
ADF, %	28.1	28.5	26.7	29.3	28.5	9.7%
NDF, %	51.4	54.4	54.7	60.5	57.3	17.7%
ADL, %	4.2	3.8	3.4	3.0	3.8	40.0%
ADL/NDF Ratio, % NDF	8.1	7.0	6.3	4.9	6.5	65.3%
Sugar, %	6.3	5.8	7.3	7.0	5.7	28.1%
Starch, %	3.7	4.0	5.1	4.2	4.9	37.8%

[1] % change between lowest and highest values in days regrowth

Table 3. Experiment 1 and 2 average CH_4 1300-1600 h and predicted CH_4 (mmol/d). Methane was measured and averaged over 1300-1600 h for Tifton 85 bermudagrass and Marshall annual ryegrass at 14 d, 21 d, 28 d, 35 d, and 42 d regrowth. Means were calculated by LS means and data were reported as significant of *P <0.05.* Methane production was also estimated using statistical equations from Mills et al. (2003) or Moe and Tyrrell as cited by Ellis et al. (2007).

Tifton 85 Bermudagrass

	14 d	**21 d**	**28 d**	**35 d**	**42 d**	**SE**	***P-value***
CH_4 predicted (mmol/d)[1]	38.40	37.57	35.32	36.44	35.59		
CH_4 predicted (mmol/d)[2]	39.52	41.93	43.23	47.35	44.20		
CH_4 measured (mmol/d)	19.07^{c}	22.89^{bc}	32.13^{a}	27.00^{ab}	23.89^{bc}	2.53	0.0007
Marshall Annual Ryegrass							
CH_4 predicted (mmol/d)[1]	34.78	41.37	40.64	42.31	34.84		
CH_4 predicted (mmol/d)[2]	35.33	31.66	27.78	30.04	30.73		
CH_4 measured (mmol/d)*	14.46^{b}	17.21^{a}	11.61^{c}	8.14^{d}	10.93^{c}	1.50	<0.0001

[1]CH4 predicted by modified Mills et al. (2003)
[2]CH4 predicted by modified Moe and Tyrrell as cited by Ellis et al. (2007)
*Significant treatment*time interaction

Table 4. Experiment 1 and 2 average CH_4 per g DM 1300-1600 h (mmol/d per g DM apparently digested or per g NDF-D) for Tifton 85 bermudagrass and Marshall annual ryegrass at 14 d, 21 d, 28 d, 35 d, and 42 d regrowth. Means were calculated by LS means and data were reported as significant if *P<0.05.*

Tifton 85 Bermudagrass

	14 d	**21 d**	**28 d**	**35 d**	**42 d**	**SE**	***P-value***
CH_4 (mmol/g NDF-D)	4.50[a]	2.36[b]	3.02[b]	2.54[b]	2.37[b]	0.44	<0.0001
CH_4 (mmol/g DM apparent digested)	2.13[a]	1.38[c]	1.94[ab]	1.90[ab]	1.56[bc]	0.23	0.0124

Marshall Annual Ryegrass

CH_4 (mmol/g NDF-D)*	2.47[b]	3.34[a]	2.31[b]	1.50[d]	1.91[c]	0.25	<0.0001
CH_4 (mmol/g DM apparent digested)*	0.87[b]	1.10[a]	0.73[c]	0.52[d]	0.70[c]	0.10	<0.0001

*Significant treatment*time interaction

Table 5. Experiment 1 and 2 nutrient (g DM fed) and CH_4 (mmol/d) correlations. The nutrients starch, sugar, NDF, ADF, ADL, HC, C, and CP in Tifton 85 bermudagrass and Marshall annual ryegrass were correlated with CH_4 (n=20). Correlations were considered significant if *P<0.05* and trends were considered if *P<0.10.*

Variable	Bermudagrass		Annual Ryegrass	
	CC[1]	*P*-value	CC[1]	*P*-value
Starch	0.792	<0.0001	0.882	<0.0001
Sugar	0.777	<0.0001	0.497	0.0259
NDF	0.542	0.0136	0.387	0.0918
ADF	-0.063	0.7927	0.041	0.8643
ADL	-0.706	0.0005	- 0.109	0.6485
HC[2]	0.676	0.0011	0.704	0.0005
C[3]	0.169	0.4766	0.073	0.7589
CP	-0.695	0.0007	-0.184	0.4363

[1]Correlation coefficients from PROC CORR (SAS Institute)
[2]Hemicellulose
[3]Cellulose

Table 6. Experiment 1 forward stepwise regression when feeding Tifton 85 bermudagrass (n=20) (g DM fed per d) for the estimation of CH_4 production (mmol/d). Variables were added to the model and those that met a significance level of 0.10 and had a variance inflation factor (VIF) of <10 were included in the model.

Step	Intercept	Starch	Sugar	ADL	Model R^2	*P-value*
1	-1.92	21.85			0.628	<0.001
2	-16.80	15.83	12.37		0.881	0.03
3	-1.25	15.28	9.20	-8.98	0.902	0.09
VIF		1.21	2.17	2.12		

Table 7. Experiment 2 volatile fatty acids (mol/100 mol). The fermentation profile when feeding Marshall annual ryegrass at 14 d, 21 d, 28 d, 35 d, and 42 d regrowth at 0, 2 and 4 h after feeding. Means were calculated by LS means and data was reported significant if *P<0.05.*

	Ryegrass						*P-value*		
VFA	14 d	21 d	28 d	35 d	42 d	SE	Diet	Time	Diet*time
Acetate						1.22	0.0058	<0.0001	0.0045
0 h	57.9^{gh}	64.9^{ab}	59.1^{fgh}	60.3^{efg}	60.8^{def}				
2 h	58.2^{gh}	65.5^{a}	62.9^{bcd}	64.0^{abc}	62.3^{cde}				
4 h	54.3^{i}	64.0^{abc}	59.4^{fgh}	62.1^{cde}	57.5^{h}				
Propionate						0.36	0.0024	0.0383	<0.0001
0 h	24.6^{b}	22.3^{d}	22.7^{cd}	27.0^{a}	24.6^{b}				
2 h	28.4^{a}	22.2^{d}	22.4^{d}	23.9^{bc}	25.3^{b}				
4 h	28.1^{a}	22.8^{cd}	22.7^{cd}	24.4^{b}	27.2^{a}				
Isobutyrate						0.12	0.001	<0.0001	0.0002
0 h	0.6^{j}	1.1^{f}	1.5^{ab}	1.3^{cd}	0.9^{gh}				
2 h	0.3^{k}	1.0^{fg}	1.1^{ef}	1.5^{ab}	0.7^{ij}				
4 h	0.4^{k}	1.2^{de}	1.4^{bcd}	1.6^{a}	0.8^{hi}				
Butyrate						0.56	0.0121	<0.0001	0.0168
0 h	11.4^{ab}	8.2^{efg}	10.4^{bc}	6.9^{gh}	8.8^{ef}				
2 h	9.5^{cde}	8.0^{fg}	9.5^{def}	6.3^{h}	8.1^{efg}				
4 h	12.3^{a}	8.3^{efg}	11.5^{a}	6.9^{gh}	9.9^{bcd}				
Isovalerate						0.25	0.0074	0.01112	0.0012
0 h	1.3^{de}	1.8^{bc}	2.6^{a}	2.1^{b}	1.5^{cd}				
2 h	0.8^{g}	1.9^{bc}	1.9^{bc}	2.4^{a}	1.2^{efg}				
4 h	0.9^{fg}	1.9^{bc}	2.2^{ab}	2.7^{a}	1.2^{def}				
Valerate						0.39	0.0448	<0.0001	0.1231
0 h	4.2^{a}	1.9^{ef}	3.9^{ab}	2.5^{cdef}	3.2^{bcd}				
2 h	2.7^{cde}	1.6^{f}	2.2^{def}	1.9^{ef}	2.3^{def}				
4 h	4.0^{ab}	1.9^{ef}	2.9^{cde}	2.4^{cdef}	3.2^{abc}				
Acetate:Propionate						0.07	0.0018	0.0005	0.0002
0 h	2.4^{gf}	3.0^{a}	2.6^{ed}	2.3^{gh}	2.5^{ef}				
2 h	2.1^{hi}	2.9^{a}	2.8^{ab}	2.7^{bcd}	2.5^{ef}				
4 h	1.9^{i}	2.8^{abc}	2.6^{cde}	2.6^{def}	2.1^{hi}				
pH						0.003	0.0107	0.0001	0.0245
0 h	6.6^{abc}	6.4^{a}	6.6^{ab}	6.6^{ab}	6.6^{ab}				
2 h	6.2^{f}	6.6^{ab}	6.5^{bcd}	6.6^{abc}	6.4^{de}				
4 h	6.3^{ef}	6.6^{ab}	6.5^{abcd}	6.6^{ab}	6.5^{cd}				

Table 8. Experiment 2 nutrient content of Marshall annual ryegrass (DM basis unless otherwise stated) for grasses of 14 d, 21 d, 28 d, 35 d, and 42 d regrowth. The analyses were run as single samples without statistics. All analyses were done at Cumberland Valley Analytical Laboratories in Hagerstown, MD.

Item	Ryegrass by Days Regrowth					
	14-d	**21-d**	**28-d**	**35-d**	**42-d**	**%Change**[1]
DM, %	100.0	92.3	91.7	91.2	92.1	9.6%
CP, %	17.3	32.1	36.4	32.4	24.8	110.0%
Soluble Protein, % CP	38.9	37.2	44.2	29.1	34.6	51.9%
Rumen Degradable Protein, % CP	69.4	68.6	72.1	64.6	67.3	11.6%
ADF, %	23.7	20.9	16.9	24.2	19.7	43.2%
NDF, %	38.1	37.7	31.0	36.8	36.5	22.9%
ADL, %	3.2	2.7	2.0	3.5	2.6	75.0%
ADL/NDF Ratio, % NDF	8.4	7.2	6.4	10.2	7.2	59.4%
Sugar, %	13.8	8.1	11.4	2.6	9.7	431.0%
Starch, %	1.9	2.5	2.0	1.0	1.1	150.0%

[1] % change between lowest and highest values in days regrowth

Table 9. Experiment 2 forward stepwise regression when feeding Marshall annual ryegrass (n=20) (g DM fed per d) for estimation of CH_4 production (mmol/d). Variables were added to the model and those that met a significance level of 0.10 and had a variance inflation factor (VIF) of <10 were included in the model.

Variables	**Intercept**	**Starch**	**HC**	**ADL**	**Model R^2**	***P-value***
1	4.27	17.23			0.777	<0.0001
2	-5.75	13.89	2.77		0.910	<0.0001
3	-9.64	14.60	2.93	3.64	0.939	0.0135
VIF		1.27	1.24	1.09		

Chapter 2 Figures

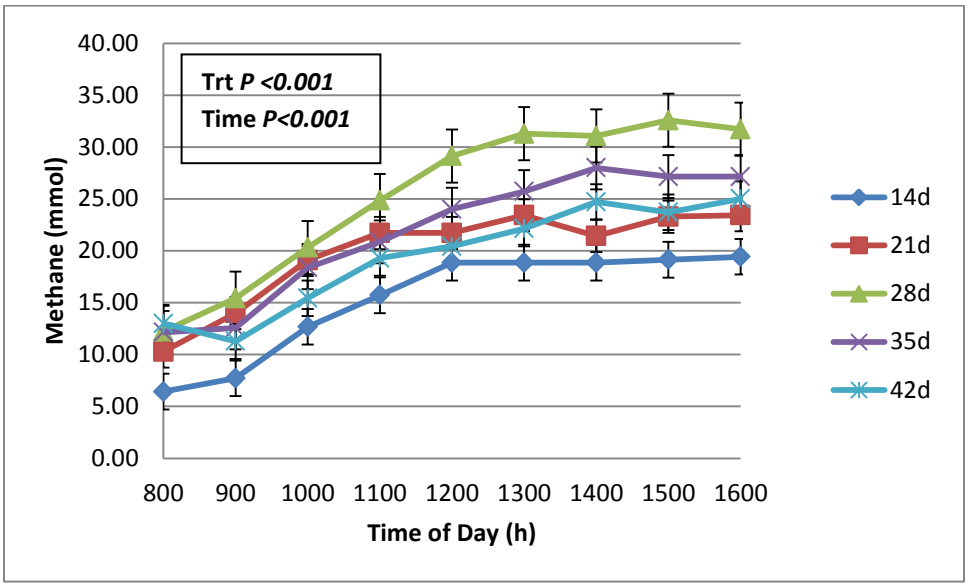


Figure 1: Experiment 1 hourly CH_4 production when feeding Tifton 85 bermudagrass at 14 d, 21 d, 28d, 35 d, and 42 d regrowth from 0800-1600 h averaged across all sampling days. Continuous cultures were fed at 0800 h and 1600 h, and means were calculated by LS means and differences were significant if *P<0.05.*

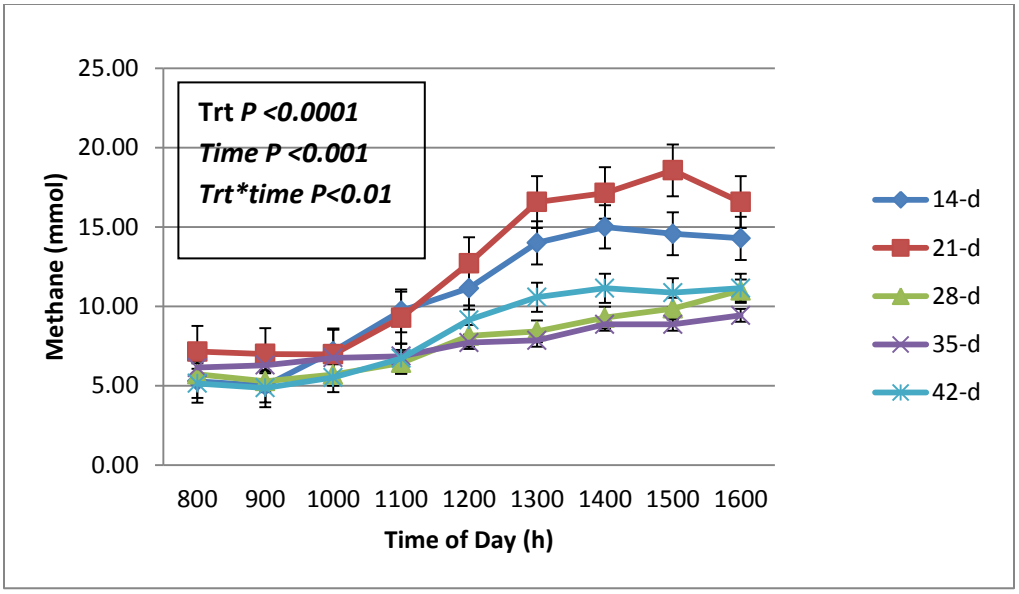
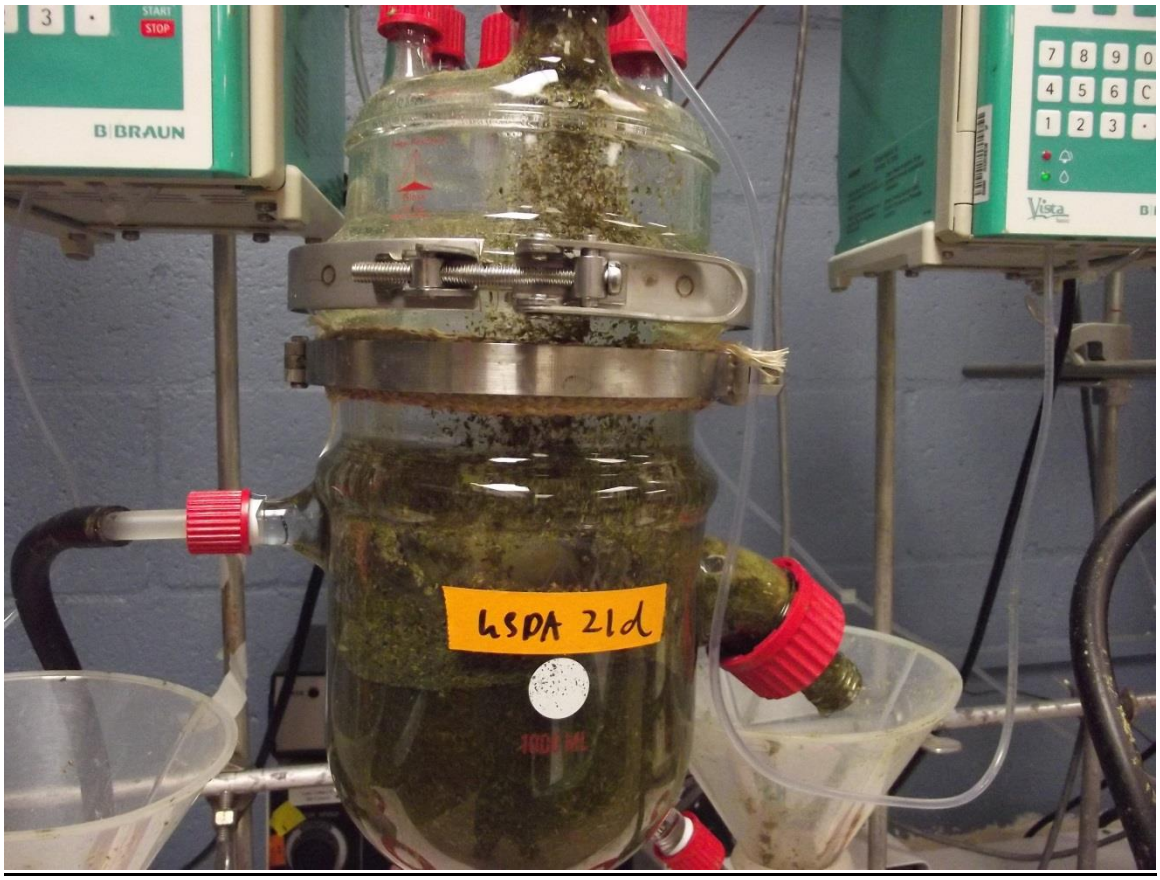


Figure 2: Experiment 2 hourly CH_4 production when feeding Marshall annual ryegrass at 14 d, 21 d, 28d, 35 d, and 42 d regrowth from 0800-1600 h averaged across all sampling days. Continuous cultures were fed at 0800 h and 1600 h, and means were calculated by LS means and differences were significant if *P<0.05.*

APPENDICIES

Appedix A

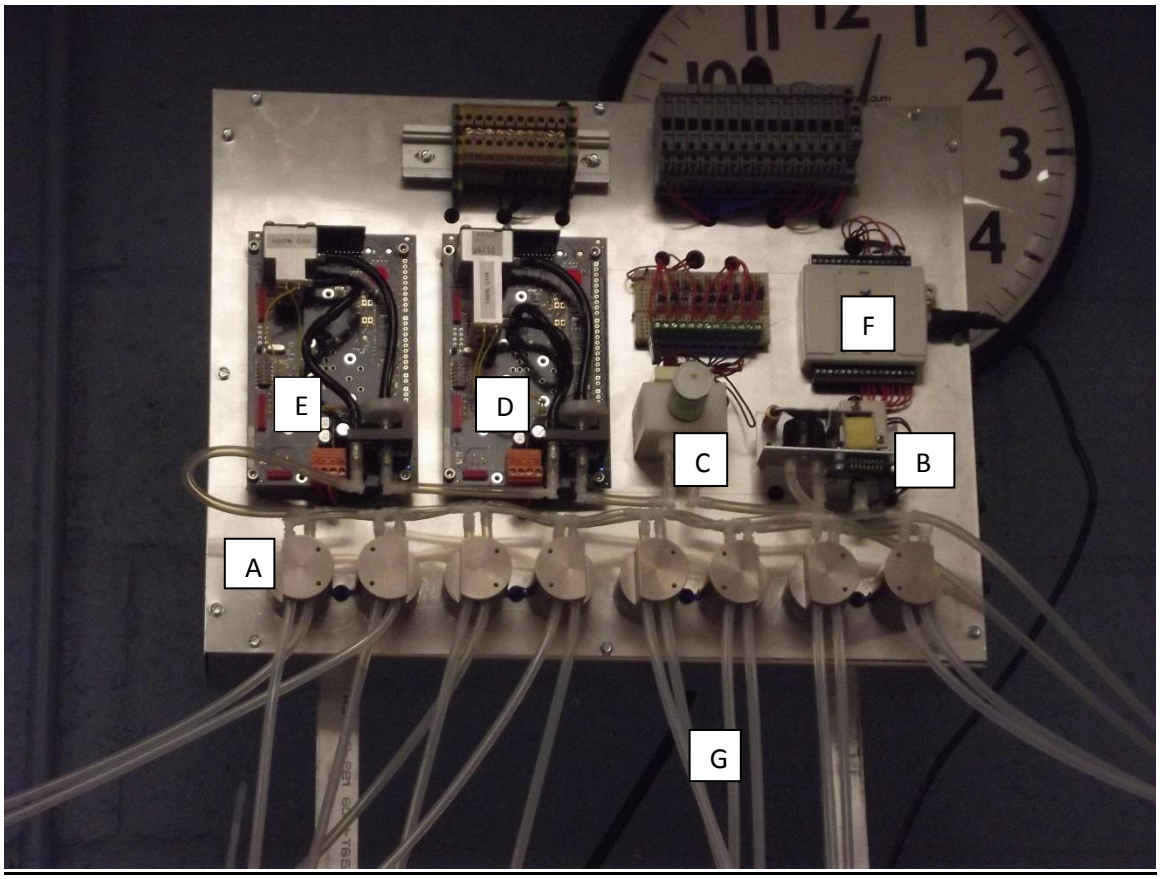
Ruminal Continuous Culture



Example Tifton 85 Bermudagrass fed at 21 d regrowth

Appendix B

Gas Sensor System to Measure CH_4, CO_2, and O_2



With A) two-way pinch valves, B) air pump, C) O_2 sensor, D) CH_4 sensor, E) CO_2 sensor, F) data acquisitioner and G) silica tubing

Appendix C

Steps for CH_4 and CO_2 Gas Sensor Calibration

1. Sensor box was powered up for 30 minutes prior to calibration.

2. A tank of nitrogen was used as the zero gas and was run through each sensor at a flow rate of 1000 mL/minute for one minute. Once readings stabilized, sensor readings were adjusted to zero by using the + or – buttons labeled “zero” on each sensor.

3. A span gas (100% CH_4 or CO_2) was used for each sensor at a flow rate of 1000 mL/min for one minute. Once readings stabilized, sensor readings were adjusted to 100% by using the + or – buttons labeled “span” on each sensor.

4. Readings were checked after calibration using a specialty gas mixture (20% CH_4, 5% O_2, balanced with CO_2, accuracy +/- 2%) run through at a flow rate of at least 200 mL/minute.

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